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Disrupted fat absorption attenuates obesity induced by a high-fat diet in *Clock* mutant mice

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Abstract **The *Clock* gene is a core component of the circadian clock in mammals. We show here that serum levels of triglyceride and free fatty acid were significantly lower in circadian *Clock* mutant ICR than in wild-type control mice, whereas total cholesterol and glucose levels did not differ. Moreover, an increase in body weight induced by a high-fat diet was attenuated in homozygous *Clock* mutant mice. We also found that dietary fat absorption was extremely impaired in *Clock* mutant mice. Circadian expressions of cholecystokinin-A (CCK-A) receptor and lipase mRNAs were damped in the pancreas of *Clock* mutant mice. We therefore showed that a *Clock* mutation attenuates obesity induced by a high-fat diet in mice with an ICR background through impaired dietary fat absorption. Our results suggest that circadian clock molecules play an important role in lipid homeostasis in mammals.**

Keywords: Circadian rhythm; *Clock*; Obesity; Lipogenesis; Lipid digestion; Cholecystokinin-A receptor

1. Introduction

Endogenous oscillators control most physiological circadian rhythms including those associated with food digestion and absorption [1]. Recent elucidation of the molecular mechanism of the circadian clock [2,3] has revealed that CLOCK is a core componen of circadian oscillators both in *Drosophila* and in mammals. *Clock* was the first clock gene identified in vertebrates by forward mutagenesis using *N*-ethyl-*N*-nitrosourea in a behavioral screening [4]. The free-running rhythm of locomotor activity is increased by about 4 h in homozygous *Clock* mutant mice with an ICR background in constant darkness [5]. Although murine circadian behavior can be entrained to an environmental light–dark (LD) cycle, locomotor activity, body temperature, and wake–sleep rhythms of *Clock* mutant ICR mice are phase-delayed for several hours [6]. The *Clock* gene encodes a basic helix–loop–helix (bHLH)-PAS transcription factor [2,3]. We used microarray technology to identify putative CLOCK target genes in the mouse liver that encode key physiological molecules that are related to lipid metabolism, gluconeogenesis, immune functions and the cell cycle [7]. These results suggested that in addition to being a core component of the circadian oscillator, CLOCK is involved in various physiological functions.

The present study examines the effect of a *Clock* gene mutation on murine weight control. We found that the increase in body weight induced by a high-fat diet is attenuated in homozygous *Clock* mutant mice. Furthermore, dietary fat absorption is defective in *Clock* mutants, perhaps partly due to the decrease in pancreatic expression levels of the cholecystokinin-A (CCK-A) receptor in *Clock* mutant mice. Our results suggest that circadian clock molecules play an important role in lipid homeostasis in mammals.

2. Materials and methods

2.1. Animals

Clock mutants were derived from mice supplied by J.S. Takahashi (Northwestern University, Evanston, IL) that originally had the *Clock* allele on BALB/c and C57BL/6J backgrounds [5,6]. A breeding colony was established by further backcrossing with Jcl:ICR mice. Male mice were maintained under a 12:12 h light–dark cycle (lights on at 00:00 and lights off at 12:00).

We examined the effect of a high-fat diet as follows. Eight-week-old mice were fed with normal chow (CE-2; Clea Japan Inc.) or with a high-fat diet (32% safflower oil, 20% casein, 0.3% DL-methionine, 10% sucrose, 28% corn starch, 1% vitamin mixture, 3.5% mineral mixture and 5% cellulose powder) for 7 weeks.

Dietary fat absorption was tested as follows. Nine-week-old mice that consumed a normal chow diet were fasted overnight and given 10 ml/kg of olive oil (Sigma, St. Louis, MO) p.o. at 02:00 on the following morning. Serum levels of triglyceride (TG) and free fatty acid (FFA) were evaluated in blood collected from the tail vein before, 3, 6, 9 and 12 h after olive oil administration. To examine post-prandial fat distribution, mice were injected with 10 μCi of [^{3}H] triolein (Perkin–Elmer Life and Analytical Sciences, Boston, MA) in olive oil after overnight food deprivation. Two hours after intragastric gavage, the stomach and small intestine were removed and rinsed with saline to obtain intraluminal contents with unabsorbed lipids. Tissues and their contents were then homogenized in saline, and radioactivity was determined by liquid scintillation counting.

2.2. Measurement of serum humoral factors

Mouse blood was centrifuged for 10 min at maximum speed in a desktop centrifuge. Serum samples were collected and stored at −80 °C. Serum TG, FFA, total cholesterol, and glucose levels were measured using kits (Wako Pure Chemical Industries Ltd., Osaka,

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Japan). Serum insulin and leptin levels were measured using Mercodia Mouse Insulin ELISA (Mercodia AB, Sweden) and Mouse Leptin ELISA (Morinaga Institute of Biological Science, Yokohama, Japan) kits, respectively.

2.3. Northern blotting

Total RNA was extracted from pancreas using guanidinium thiocyanate followed by ISOGEN (Nippon Gene Co., Ltd., Japan). Total RNA (20 μg) from tissues at each time point was denatured, separated on 1% agarose/0.7 M formaldehyde gels and blotted onto nylon membranes (GeneScreen Plus; DuPont, USA) by passive capillary transfer. The probes generated from cDNA fragments of *CCK-A receptor* (bases, 10261–11023; GenBank accession number D85605), *lipase* (bases, 401–1257; GenBank accession number NM_026925) and *GAPDH* (bases, 133–575; GenBank accession number M17701) were hybridized and detected as described [8]. Samples were normalized against the corresponding *GAPDH* RNA levels.

3. Results and discussion

In our previous report [7], we described that *Clock* gene mutation affects the hepatic mRNA expression of several key metabolic enzymes in mice. To determine the effect of *Clock* gene mutation on lipid homeostasis, we evaluated metabolic parameters in homozygous *Clock* mutant mice. Levels of serum TG and FFA showed circadian rhythms in both wild-type and *Clock* mutant mice, although the circadian phase was delayed for several hours in the mutants (Fig. 1A and B). A circadian phase delay was also found in locomotor activity, sleep-wake cycles, body temperature and peripheral circadian gene expression in *Clock* mutant mice [6,7]. However, the present study found that both TG and FFA levels were significantly lower in *Clock* mutant, than in wild-type mice

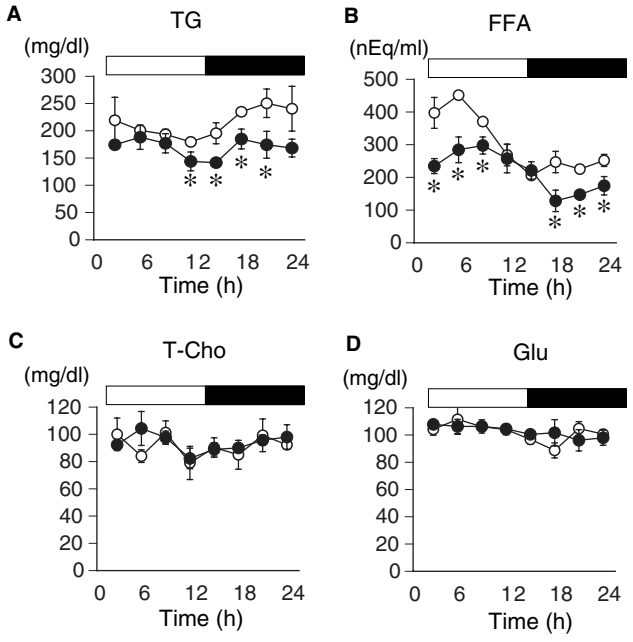

Fig. 1. Circadian profiles of metabolic parameters. Daily fluctuations in serum (A) triglycerides (TG), (B) free fatty acids (FFA), (C) total cholesterol (T-Cho) and (D) glucose (Glu) levels in homozygous *Clock* mutant mice. Open and solid bars indicate lights-on and off, respectively. Open and closed circles indicate wild-type and homozygous *Clock* mutant mice, respectively. Values are means ± S.E. ($n = 6$). At each time point, genotypes were compared using independent sample *t*-tests ($^{*}P < 0.05$).

throughout the day (Fig. 1A and B), although total cholesterol and glucose levels were identical (Fig. 1C and D). Serum levels of TG and FFA are regulated by complex mechanisms that include lipogenesis and lipolysis. We previously described that the hepatic expression of lipogenic enzymes such as fatty acid synthase (FAS), acyl-CoA synthase 4 (ACS4) and long chain fatty acyl elongase (Lce) is reduced in *Clock* mutant mice [7]. The circadian expression of lipogenic key enzymes such as acetyl-CoA carboxylase (ACC) and ATP-citrate lyase (ACL) is also considerably weakened in the liver of *Clock* mutant mice (unpublished data). These observations suggest that the reduced levels of serum TG and FFA in *Clock* mutant mice (Fig. 1A and B) were partly caused by impaired hepatic lipogenesis. Shimba et al. recently showed that BMAL1 overexpression induces the transcription of these lipogenetic enzymes in 3T3-L1 adipocytes [9]. Sterol regulatory element binding protein 1 (SREBP-1) regulates the transcription of several factors required for lipogenesis such as FAS, ACC, and ACL [10], and the circadian mRNA expression of *SREBP-1* is diminished in *Clock* mutant mice (unpublished data). Chromatin immunoprecipitation and in vitro reporter assays of the promoter region of the *SREBP-1* gene have revealed that CLOCK and BMAL1 actually transactivate this gene [9]. Taken together, these observations suggest that CLOCK and BMAL1, which are core components of the circadian clock, are directly or indirectly involved in mammalian lipogenesis.

We then compared the body weight (BW) of *Clock* mutant and wild-type mice. The post-natal BW of mice increased to a slightly lesser extent in *Clock* mutant, than in wild-type mice, although the BW of newborn animals did not differ between the genotypes (data not shown). However, the mice weighed the same when fed with normal chow after 13 weeks of age (Fig. 2A). Feeding the animals with a high-fat diet for 7 weeks from the age of 8 weeks caused a considerable increase in the BW of wild-type, compared with *Clock* mutant mice (Fig. 2B). In contrast, the gain in BW was similar in *Clock* mutant and wild-type mice fed with normal chow (Fig. 2A). The attenuated increase in the BW of *Clock* mutant mice fed with the high-fat diet was probably not due to a reduction in appetite, because the *Clock* mutation increased, rather than decreased food intake (Fig. 2D). Serum insulin and leptin levels did not differ between the genotypes when the mice were fed with the normal diet (Fig. 2E and F). Furthermore, hyperinsulinemia and hyperleptinemia induced by the high-fat diet were rather suppressed than enhanced in *Clock* mutant mice, suggesting that obesity induced by the high-fat diet was suppressed through action not only on the BW increase but also on the biochemical markers that are hallmarks of metabolic disease.

We therefore postulated that the *Clock* mutation attenuates dietary fat digestion and/or absorption in mice. To test this hypothesis, we administered the mice with olive oil and evaluated fat absorption in vivo (Fig. 3A and B). Serum increases in TG and FFA levels were obviously suppressed after olive oil administration in *Clock* mutant mice, whereas those in wild-type mice increased immediately after administration. Serum FFA might be derived from cytoplasmic TG, de novo lipogenesis, or TGs generated from dietary lipoproteins. We confirmed that lipid absorption is impaired in *Clock* mutant mice by administering them with [^{3}H]-triolein (Fig. 3C). The majority of recovered radioactivity was found in small intestine contents (25%) and intestinal wall (22%) in wild-type mice,

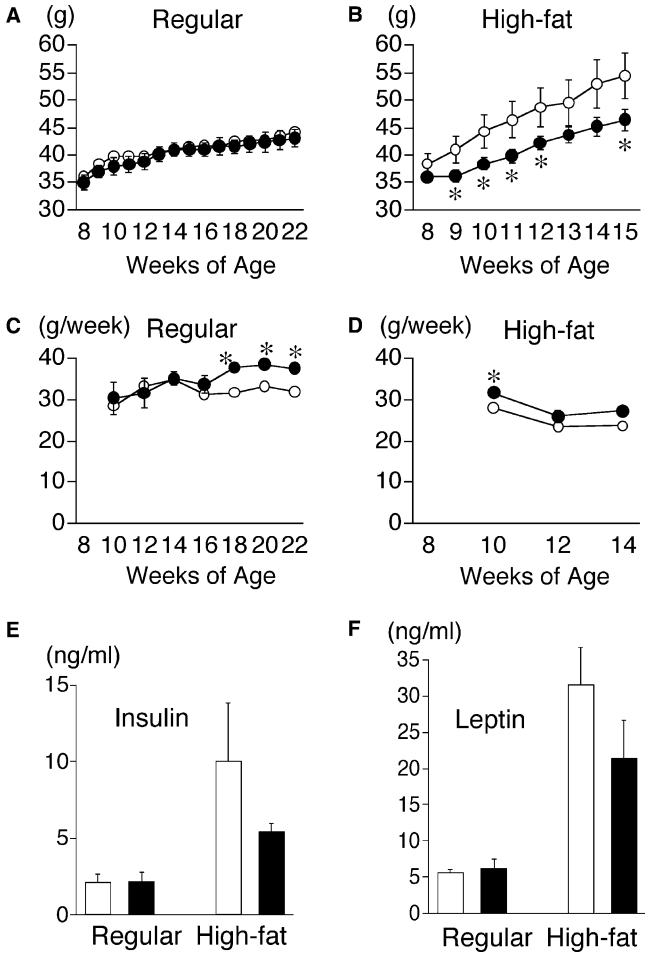

Fig. 2. *Clock* mutation attenuates high-fat diet-induced obesity. Eight-week-old wild-type (A, C, E) and *Clock* mutant (B, D, F) mice were fed with normal chow or high-fat diets for 7 weeks. (A and B) Gain in BW; (C and D) weekly food intake; (E and F) serum insulin and leptin levels Open and closed circles/bars indicate wild-type and homozygous *Clock* mutant mice, respectively. Values are means ± S.E. ($n = 6$). Genotypes were compared by independent sample *t*-tests ($^*P < 0.05$).

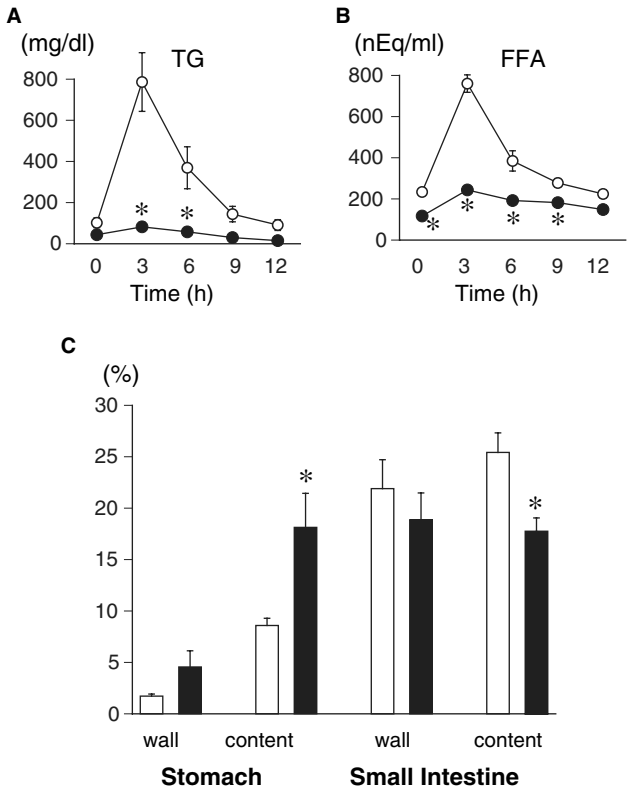

Fig. 3. Disrupted fat absorption in *Clock* mutant mice. (A and B) Post-prandial TG and FFA absorption. Mice that consumed a normal chow diet were fasted overnight and given 10 ml/kg of olive oil p.o. at 02:00 on the following morning. (C) Radioactivity recovered from gastrointestinal tract of mice given an intragastric load of [^{3}H] triolein. After fasting overnight, mice were injected with 10 μCi of [^{3}H] triolein in olive oil. Two hours after intragastric gavage intraluminal contents with unabsorbed lipids were obtained from stomach and small intestine homogenates. Radioactivity was determined by liquid scintillation counting. Open and closed circles/bars indicate wild-type and homozygous *Clock* mutant mice, respectively. Values are means ± S.E. ($n = 7$). Genotypes were compared by independent sample *t*-tests ($^*P < 0.05$).

while the radioactivity was 18% and 19%, respectively, in *Clock* mutant mice. Furthermore, 18% of radioactivity was remained in stomach contents in *Clock* mutant mice, although only 9% of the radioactivity was remained in wild-type mice.

Triolein absorption requires digestion followed by actual absorption. We found a relatively large amount of radioactivity in the stomach contents in *Clock* mutant mice, suggesting that lipid digestion rather than absorption was disrupted by the *Clock* mutation. One cause could be impaired secretion of pancreatic lipolytic enzymes such as triglyceride lipase, lipase-related proteins 1 and 2, colipase and cholesterol ester lipase. Fig. 4 also shows that the expression of pancreatic lipase mRNA was impaired in *Clock* mutant mice. However, a decrease in *lipase* mRNA expression alone probably does not explain the impaired lipid absorption in *Clock* mutant mice since lipid absorption is virtually intact in pancreatic triglyceride lipase-deficient mice [11].

Cholecystokinin is a peptide mediator of pancreatic enzyme secretion through a specific membrane-spanning CCK-A receptor [12,13] that is important for pancreatic exocrine secretion, but not essential for maintaining either the glucose concentration or pancreatic growth in mice [14]. Intestinal CCK secretion is stimulated by long-chain fatty acids [12]. We examined the expression levels of *CCK-A receptor* mRNA in the pancreas of *Clock* mutant mice. Fig. 4 shows that pancreatic mRNA expression levels of CCK-A receptor fluctuated between 14:00 and 2:00 in wild-type mice. Moreover, the mRNA expression levels were significantly lower in *Clock* mutant, than in wild-type mice. On the other hand, the intestinal mRNA expression of *CCK* did not significantly differ between the genotypes (data not shown). The impaired absorption of dietary fat in *Clock* mutant mice loaded with olive oil might be partly due to a decrease in the expression of pancreatic CCK-A receptors.

Turek et al. [15] recently reported that *Clock* mutant mice with a C57BL/6J background are obese and develop a metabolic syndrome consisting of hyperleptinemia, hyperlipidemia, hyperglycemia and hyperinsulinemia. However, serum levels of insulin and leptin did not differ between wild-type and *Clock* mutant mice in the present study (Fig. 2E and F). Furthermore, high-fat diet-induced increases in both insulin and leptin levels were rather suppressed in *Clock* mutant mice (Fig. 2E and F). Turek et al. found that corticosterone levels are lower

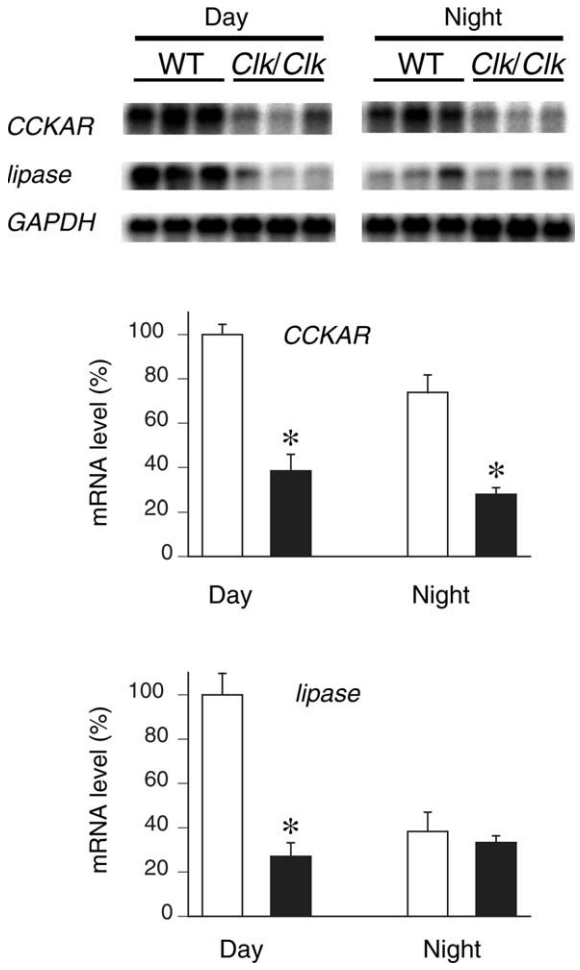

Fig. 4. Pancreatic expression of CCK-A receptor and lipase mRNAs is diminished in *Clock* mutant mice. Animals were killed at time points indicated above blots. Messenger RNA levels of genes were quantified from Northern blots ($n = 3$ each). Maximal value for wild-type mice is expressed as 100%. Open and filled bars indicate values for wild-type and homozygous *Clock* mutant mice, respectively. Values are means ± S.E. Genotypes were compared by independent sample *t*-tests ($^{*}P < 0.05$).

in *Clock* mutant mice over 24 h whereas we found no differences between the genotypes, although the circadian phase was delayed for several hours in *Clock* mutant mice (unpublished data). The *Clock* mutation thus appears to enhance and attenuate obesity between the C57BL/6J and ICR backgrounds respectively, indicating contradictory effects on the energy balance in these strains. In fact, we noted strain differences between C57BL/6J [15] and ICR (Fig. 1C and D) mice with respect to serum cholesterol and glucose levels. In fact, plasma cholesterol accumulation also differs among strains [16]. We could not exclude the possibility that CLOCK protein affects metabolic homeostasis in combination with other strain-specific loci in the mouse genome [17]. Defective dietary fat absorption in *Clock* mutant ICR mice might be unfavorable under situations such as starvation. Understanding the mechanisms underlying the present findings should lead to the identification of novel pharmaceutical targets for the treatment of obesity or hyperglycemia and for normal individuals exposed to high-fat diets.

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References

[1] Panda, S. et al. (2002) Coordinated transcription of key pathways in the mouse by the circadian clock. Cell 109, 307–320.
[2] Dunlap, J.C. (1999) Molecular bases for circadian clocks. Cell 96, 271–290.
[3] Reppert, S.M. and Weaver, D.R. (2001) Molecular analysis of mammalian circadian rhythms. Annu. Rev. Physiol. 63, 647–676.
[4] Vitaterna, M.H. et al. (1994) Mutagenesis and mapping of a mouse gene, Clock, essential for circadian behavior. Science 264, 719–725.
[5] Oishi, K., Miyazaki, K. and Ishida, N. (2002) Functional CLOCK is not involved in the entrainment of peripheral clocks to the restricted feeding: entrainable expression of mPer2 and BMAL1 mRNAs in the heart of Clock mutant mice on Jcl:ICR background. Biochem. Biophys. Res. Commun. 298, 198–202.
[6] Sei, H., Oishi, K., Morita, Y. and Ishida, N. (2001) Mouse model for morningness/eveningness. Neuroreport 12, 1461–1464.
[7] Oishi, K. et al. (2003) Genome-wide expression analysis of mouse liver reveals CLOCK-regulated circadian output genes. J. Biol. Chem. 278, 41519–41527.
[8] Oishi, K., Ohkura, N., Amagai, N. and Ishida, N. (2005) Involvement of circadian clock gene Clock in diabetes-induced circadian augmentation of plasminogen activator inhibitor-1 (PAI-1) expression in the mouse heart. FEBS Lett. 579, 3555–3559.
[9] Shimba, S. et al. (2005) Brain and muscle Arnt-like protein-1 (BMAL1), a component of the molecular clock, regulates adipogenesis. Proc. Natl. Acad. Sci. USA 102, 12071–12076.
[10] Horton, J.D. (2002) Sterol regulatory element-binding proteins: transcriptional activators of lipid synthesis. Biochem. Soc. Trans. 30, 1091–1095.
[11] Huggins, K.W., Camarota, L.M., Howles, P.N. and Hui, D.Y. (2003) Pancreatic triglyceride lipase deficiency minimally affects dietary fat absorption but dramatically decreases dietary cholesterol absorption in mice. J. Biol. Chem. 278, 42899–42905.
[12] Beglinger, C. and Degen, L. (2004) Fat in the intestine as a regulator of appetite-role of CCK. Physiol. Behav. 83, 617–621.
[13] Owyang, C. and Logsdon, C.D. (2004) New insights into neurohormonal regulation of pancreatic secretion. Gastroenterology 127, 957–969.
[14] Takiguchi, S. et al. (2002) Role of CCK-A receptor for pancreatic function in mice: a study in CCK-A receptor knockout mice. Pancreas 24, 276–283.
[15] Turek, F.W. et al. (2005) Obesity and metabolic syndrome in circadian Clock mutant mice. Science 308, 1043–1045.
[16] Schwarz, M., Davis, D.L., Vick, B.R. and Russell, D.W. (2001) Genetic analysis of cholesterol accumulation in inbred mice. J. Lipid Res. 42, 1812–1819.
[17] Shimomura, K. et al. (2001) Genome-wide epistatic interaction analysis reveals complex genetic determinants of circadian behavior in mice. Genome Res. 11, 959–980.